Adhesion of *Porphyromonas gingivalis* and *Tannerella forsythia* to dentin and titanium with sandblasted and acid etched surface coated with serum and serum proteins - an *in vitro* study

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Running title: Adhesion of *P. gingivalis* and *T. forsythia*

Highlights

- *Tannerella forsythia* adheres more to a titanium SLA surface than to dentin.
- Adhesion of a multi-species biofilm is not remarkably influenced by the substrate.

- Titanium SLA inhibits expression of *Tannerella forsythia* protease inhibitor.

Abstract

Objective: To evaluate the adhesion of selected bacterial strains incl. expression of important virulence factors at dentin and titanium SLA surfaces coated with layers of serum proteins.

Methods: Dentin- and moderately rough SLA titanium-discs were coated overnight with human serum, or IgG, or human serum albumin (HSA). Thereafter, *Porphyromonas gingivalis, Tannerella forsythia,* or a six-species mixture were added for 4 h and 24 h. The number of adhered bacteria (colony forming units; CFU) was determined. Arg-gingipain activity of *P. gingivalis* and mRNA expressions of *P. gingivalis* and *T. forsythia* proteases and *T. forsythia* protease inhibitor were measured.

Results: Coating specimens never resulted in differences exceeding 1.1 log10 CFU, comparing to controls, irrespective the substrate. Counts of *T. forsythia* were statistically significantly higher at titanium than dentin, the difference was up to 3.7 log10 CFU after 24 h ($p=0.002$). No statistically significant variation regarding adhesion of the mixed culture was detected between surfaces or among coatings. Arg-gingipain activity of *P. gingivalis* was associated with log10 CFU but not with the surface or the coating. Titanium negatively influenced mRNA expression of *T. forsythia* protease inhibitor at 24 h ($p=0.026$ uncoated, $p=0.009$ with serum).

Conclusions: The present findings indicate that: a) single bacterial species (*T. forsythia*) can adhere more readily to titanium SLA than to dentin, b) low expression of *T. forsythia* protease inhibitor may influence the virulence of the species on titanium SLA surfaces in comparison with teeth, and c) surface properties (e.g. material and/or protein layers) do not appear to significantly influence multi-species adhesion.

Key words: *Porphyromonas gingivalis; Tannerella forsythia*; bacterial proteases; implant surface; proteinaceous layer

Introduction

Teeth are continuously exposed inside the surrounding sulcus to a transudate (during homeostasis) or to an exudate during inflammation, called gingival crevicular fluid (GCF), (Griffiths, 2003). Resting volume of GCF is between 0.05 µl and 2 µl depending on the periodontal disease status (Goodson, 2003). Similarly to teeth, oral implants are surrounded by peri-implant sulcular fluid (PISF), the volume of which has been determined to be 0.5 µl in health and about 1.3 µl in disease (Arikan, Buduneli, & Lappin, 2011).

The components of GCF derive from many sources, such as serum, connective tissue and epithelium, as well as from inflammatory cells and bacteria which are present in the crevice and the surrounding soft tissues (Griffiths, 2003). Research on GCF protein concentration has suggested a composition similar to that of serum (Tew, Marshall, Burmeister, & Ranney, 1985), and serum proteins, e.g. albumin, transferrin and immunoglobulin (Ig)G are incorporated in the pellicles formed on teeth in vivo (Carlen, Rudiger, Loggner, & Olsson, 2003). Despite the fact that no exact data about the protein composition in PISF are available, it appears to be similar to GCF (Ozcakir-Tomruk, Chiquet, & Mericske-Stern, 2012).

Although most of the microbiota colonizing the oral cavity is considered being commensal, the presence of specific bacteria, like *Porphyromonas gingivalis*, *Treponema denticola, Tannerella forsythia* (i.e. “the red complex”), and *Aggregatibacter actinomycetemcomitans*, is strongly associated with an increased risk for periodontitis (Socransky, Haffajee, Cugini, Smith, & Kent, 1998). In particular, *P. gingivalis* is considered a key-stone pathogen due its ability of causing dysbiosis between the host and the oral biofilm (Hajishengallis, Darveau, & Curtis, 2012). Major virulence factors of *P. gingivalis* are its arginine-specific (HRgpA and RgpB; encoded by two genes *rgpA* and *rgpB*) and lysine-specific (Kgp, encoded by *kgp*) cysteine proteases, also called gingipains (Y. Guo, Nguyen, & Potempa, 2010). *T. forsythia* encodes also several proteases (Ksiazek, Mizgalska, Eick, et al., 2015), which alike gingipains are secreted via a novel Type IX secretion system (T9SS) (Tomek et al., 2014). Moreover it encodes a bacterial serpin (called miropin) that inhibits several host proteases (Ksiazek, Mizgalska, Enghild, et al., 2015).

In general, it appears that there are major similarities in the microbioms associated with periodontitis and peri-implantitis (Kumar, Mason, Brooker, & O'Brien, 2012). Further, it has been demonstrated, that, as it occurs on natural teeth (K. H. Lee, Tanner, Maiden, & Weber, 1999), periodontopathogens colonize implants immediately after placement (Furst, Salvi, Lang, & Persson, 2007; Quirynen et al., 2006). In this context, the initial stages of biofilm formation (Jakubovics & Kolenbrander, 2010) involve bacterial adhesion to the pellicle-coated

surfaces and it has been shown in vitro that adherence of bacteria associated with gingivitis and periodontitis is mediated by plasma proteins in the pellicle (Carlen et al., 2003). In a recent in vitro study, it has been recognized that caries associated bacteria (i.e., *Streptococcus mutans* and *Streptococcus mitis* biovar 2) exhibit a marked upregulation in their proteolytic activity when in contact with salivary proteins coated on a surface, despite very dissimilar patterns of adhesion to the same protein (Kindblom, Davies, Herzberg, Svensater, & Wickstrom, 2012). There is limited information, however, on how different protein coatings on different substrates may influence attachment and/or protease activity of bacteria associated with periodontitis and/or peri-implantitis.

Therefore, the aims of this *in vitro* study were: a) to evaluate the adhesion of single strains of *P. gingivalis, T. forsythia,* and of a six-species mixture including *P. gingivalis* and *T. forsythia* and in addition two early colonizers (*Streptococcus gordonii*, *Actinomyces naeslundii*), and two bridging species (*Fusobacterium nucleatum*, *Parvimonas micra*) of oral biofilms on dentin and titanium after having been coated with protein components of gingival crevicular fluid and peri-implant sulcular fluid, and b) to analyze the mRNA expression of major virulence factors of *P. gingivalis* and *T. forsythia* in dependence of material and serum-coating.

Material & methods

Bacterial strains

All used bacterial strains were reference strains. As single strains were tested *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037. The six-species mixture consisted of the mentioned *P. gingivalis* and *T. forsythia* strains with the addition of *S. gordonii* ATCC 10558 and *A. naeslundii* ATCC 12104 as early colonizers, and *F. nucleatum* ATCC 25586 and *P. micra* ATCC 33270 as bridging species. Before each experiment, all strains were precultivated on Schaedler agar plates (Oxoid, Basingstoke, UK) with 5% sheep blood and vitamin K addition, in an anaerobic atmosphere or with 5% CO_2 (*S. gordonii* ATCC 10558). Thereafter, suspension was prepared by adding bacteria in 0.9% w/v NaCl equal to OD_{600nm} =1.0. In case of mixed cultures, suspensions of the strains were mixed by adding 1 part of *S. gordonii* ATCC 10558 to each two parts of the other strains. Bacterial suspension was given to Wilkins-Chalgren broth (Oxoid, Basingstoke, UK) supplemented with 5 mg/l β-NAD in a ratio 1:50.

Test specimens

The test specimens were dentin discs from extracted human teeth and moderately rough titanium discs (SLA, Institute Straumann AG, Basel, Switzerland), with a diameter of 5 mm and a thickness of about 1 mm.

Extracted teeth were collected after the patients had signed an informed consent regarding using their teeth in *in vitro* experiments. According to the Ethical Commission of the Canton Bern, an approval for anonymous use of these by-pass products is not needed. After extraction, teeth were placed in chloramine solution for disinfection for 2 h. Thereafter, they were stored in 0.9% v/w NaCl solution and processed within 2–3 weeks. After crown removal, dentin slices of the appropriate size were cut with a diamond saw. Surface properties were standardized by grinding the dentin specimens with silicon carbide papers of #2400 grit size, corresponding to an abrasive particle size of 6.5 µm (Struers A/S, Ballerup, Denmark).

Coatings

Human serum (Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was heat-inactivated to exclude complement-activity and used in a 25% concentration in 0.9% w/v NaCl. IgG (Sigma-Aldrich Chemie GmbH) was diluted to 5 mg/ml and human serum albumin (Sigma-Aldrich Chemie GmbH) to 10 mg/ml, each with 0.9% w/v NaCl. The control was 0.9% w/v NaCl.

Adhesion assay

Dentin and titanium discs were placed in a 24 well plate and incubated at room temperature overnight with 25% human serum, 5 mg/ml IgG, 10 mg/ml HSA or 0.9% w/v NaCl (controls). Thereafter, 500 µl bacterial suspension mixed with Wilkins-Chalgren broth was added to each well. After incubation for 4 h or 24 h at 37°C in anaerobic conditions, the test specimens were transferred to tubes containing 100 µl of 0.9% w/v NaCl. Then, test specimens were subjected to 5 min of ultrasonication. (Preliminary tests have confirmed that by using this procedure viability of bacteria is not influenced and the overwhelming majority of bacteria is detached from the surface.) From these mixtures, in case of *P. gingivalis* one part was immediately frozen (-20°C) for later evaluation of arginine-specific amidolytic activity. Second, serial dilutions were made and defined volumes were spread on Schaedler agar plates. Agar plates were incubated in the respective atmosphere before the total counts of the colony forming units (CFU) were enumerated. Counting of CFU was performed using an Acolyte colony counter and Acolyte version 3.04 software (Symbiosis, Cambridge, UK). In addition in the multi-species mixtures, the counts of *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037 were determined by using real-time PCR as described recently (Eick, Straube, Guentsch, Pfister, & Jentsch, 2011).

Determination of the arginine-specific amidolytic activity

Samples taken from the *P. gingivalis* experiments were tested for arginine-specific amidolytic activity by using a chromogenic substance: N-α-benzoyl-DL-arginine-*p*-nitroanilide (BA*p*NA) (Sigma, St. Louis, MO, USA) with a final concentration of 2 mM in the assay buffer (0.2M Tris-HCl, 0,1M NaCl, 5 mM $CaCl_2$, pH 7.6, freshly supplemented with cysteine hydrochloride solution (10mM)) that had 20% DMSO. Ten µl of the samples were mixed with 10 µl of the substrate solution. The absorbance was read at 405 nm (37^{o}C) at 30 s intervals for 2 h by using a spectrophotometer (BioTek EL808, BioTek, Luzern, Switzerland).

mRNA expression of bacterial proteases

Expression of *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037 proteases and of *T. forsythia* protease inhibitor miropin on dentin and titanium discs coated with serum or with 0.9% w/v NaCl was evaluated. RNA was extracted by using the innuPREP RNA Mini Kit (Analytic Jena, Jena, Germany) and cDNA was generated by using the GoScript^{TM} Reverse Transcription System (Promega, Madison, WI, USA) according to the manufacturers' instructions. Thereafter, real-time PCR using GoTaq® qPCR Master Mix (Promega) was performed for the different bacterial proteases and miropin (Table 1) according to the manufacturer's recommendations. Quantification was made related to *sod* genes of the respective bacteria.

Statistical analysis

At least 6 independent samples (log10 CFU, BA*p*NA activity) were compared with ANOVA followed by post-hoc LSD (various coatings) and Student' t-test for independent samples (dentine vs. titanium, serum vs. 0.9% NaCl in mRNA expression experiments). Spearman test correlated the CFU counts with BA*p*NA activity. Software SPSS 23.0 (IBM, Chicago, IL, USA) was used.

Results

Single strains

In general, bacteria adhered well both to dentin and to titanium specimens (Table 1; Figs 1A-1C) and the various coatings had limited influence on bacterial adhesion, irrespective the substrate; any differences between tests and controls, even if statistically significant, never exceeded 1.1 log10 CFU. At 24 h, *P. gingivalis* adhered in higher counts on HSA-coated dentin

than on NaCl coated controls (p=0.041). At titanium surfaces, serum and HSA promoted the adhesion of *T. forsythia* ATCC 43037 after 4 h (p=0.019, p=0.024, respectively).

Coating seemed to influence Arg-gingipain activity of *P. gingivalis* ATCC 33277, where highest activity was measured after HSA coating of both titanium and dentin after 24 h comparing to controls (p=0.049 and p=0.033, respectively) (Fig. 1B). Also, log10 CFU correlated significantly with Arg-gingipain activity of *P. gingivalis* ATCC 33277 (R= 0.558, p=0.001) on dentin and R=0.556, p=0.001 on titanium.

On the other hand, higher log10 CFU were observed for some bacterial strains and coatings on titanium comparing to dentin. *P. gingivalis* was counted in higher quantities at titanium when being coated with serum (p=0.038) or NaCl controls (p=0.044). Irrespective coating, statistically significantly more *T. forsythia* adhesion was observed on titanium comparing to dentin (with a difference up to 3.7 log10 CFU for HSA after 24 h, p=0.002).

Six-species mixture

The total numbers of adhered bacteria (CFU) were 4.53±0.20 log10 at dentin and 4.56±0.14 log10 at titanium after 4 h and 6.56±0.10 log10 at dentin and 6.63±0.08 log10 at titanium after 24 h. In general, adhesion of the mixed culture did not depend on the coating or on the surface. The only significant difference was at titanium at 4 h, here the total counts were higher at a serum coated surface than at a NaCl control (p=0.016), however the difference was only 0.16 log10 CFU (Figs 2A, 2C, 2D).

In regard with the 6-species mixture, the various coatings basically did not influence bacterial adhesion irrespective of the substrate, and there were no noteworthy differences between the substrates. Similarly, there was no differences regarding the adhesion of single analyzed species within the mixture or the arginine-specific amidolytic activity of *P. gingivalis* (Fig. 2B), among coatings or between substrates. The arginine-specific amidolytic activity of *P. gingivalis*, however, correlated with the counts of *P. gingivalis* within the mixture at dentin (R=0.439, p<0.001) and at titanium (R=0.227, p=0.032).

mRNA expression of bacterial proteases and protease inhibitor

In general, expression of proteases appeared increased on dentin comparing to titanium discs. Coating with serum, in some cases resulted in increased and in other cases in decreased gingipain expression (Figs 3A-3C). The mean values of mRNA expression of gingipains adjusted to those of the planktonic bacteria were most clearly above 1.0, while mean values for *T. forsythia* protease expression (*mirolase*) were always below 1.0 and neither coating nor the substrate influenced significantly *mirolase* expression (Fig. 4C). In contrast, coating with serum resulted in significantly higher *miropsin-1* and *miropsin-2* expression (dentin: miropsin-1 p=0.037, miropsin-2 p=0.008; titanium: miropin-1 p=0.003) (Figs 4A, 4B). Further, mean expression values for the protease inhibitor miropin adjusted to expression of planktonic bacteria ranged between 0.11 and 27.5, and its expression at 24 hours was higher at dentin than at titanium both if coated with serum (p=0.009) or at the NaCl controls (p=0.026) (Fig. 4D).

Discussion

In this in vitro study, the influence of surface properties (material and/or protein layers) on the adhesion of bacterial strains being associated with periodontal and peri-implant diseases was investigated. Specifically, dentin or titanium disks were coated with various protein containing solutions, including serum and two serum proteins solubilized in 0.9% w/v NaCl. The used concentrations of the coatings were chosen based on an earlier assessment showing that, in severe periodontitis, GCF levels of HSA ranged between 2 and 16 mg/ml (median 11 mg/ml) and those of IgG between 1.5 and 7.8 mg/ml (median 5 mg/ml); those levels corresponded to 3.4 - 35.6% (median 20.6%) for HSA and 12.0 – 64.5% (median 46.4%) for IgG of the serum levels (Tew et al., 1985).

A titanium surface being widely used and retrospectively evaluated (Buser et al., 2012; Park et al., 2015) was chosen. Normally, the SLA surface of an implant is completely within bone. It seems thus reasonable to assume that the current findings may be relevant only for cases where the SLA surface is erroneously exposed from bone, for example in case of peri-implantitis. In this context, the effect of coating with saliva was not evaluating herein; in peri-implantitis, increased probing depths are present, thus it seems reasonable to assume that blood components are of more relevance than saliva, as also in the case of periodontitis.

Serum proteins in the pellicle act as binding sites for bacterial adhesion through specific interactions with bacterial adhesins (Choi et al., 2011; Lee, Kim, & Choe, 2001). In general, the various coatings did not influence bacterial adhesion – at a statistically significant level –

neither on dentin nor on titanium. These findings are in line with previous reports. For example, adhesion of *P. gingivalis* and *T. forsythia* did not exhibit clear differences when hydroxyapatite disks were coated with IgG, plasma, or serum albumin (Carlen et al., 2003). In another study, coating different titanium surfaces with serum albumin did not influence the adhesion of *P. gingivalis* and *F. nucleatum* but promoted adherence of *S. mutans* (Badihi Hauslich, Sela, Steinberg, Rosen, & Kohavi, 2013). In contrast, some differences regarding bacterial adhesion were observed between the two disk groups. In particular, larger numbers of *P. gingivalis* were observed on titanium surfaces than on dentin after serum or NaCl coating, while larger number of *T. forsythia* were observed on titanium comparing to dentin irrespective of the coating. Initial plaque formation depends on surface roughness, as shown after placement of missing teeth by different natural and prosthetic materials (Siegrist, Brecx, Gusberti, Joss, & Lang, 1991). Our finding of partly higher adhesion at titanium may thus also be attributed to differences in surface roughness between the two groups. The average surface roughness of the current titanium disks (with SLA surface) has been previously estimated to 1.19±0.04 µm (Kim et al., 2008), while the average surface roughness of dentin disks produced in the same way as those used herein has been estimated to 0.28±0.12 µm (Hagi et al., 2015). In a study using a split-mouth design, where titanium abutments with polished surfaces and different roughness (Ra= 0.05 – 0.21 µm) were used, spirochetes were found only on the roughest abutment (Ra= 0.21 µm), while abutments with surface roughness in the range of Ra 0.05 µm – 0.13 µm did not show any influence on microbial composition within plaque (Quirynen, Bollen, Papaioannou, Van Eldere, & van Steenberghe, 1996). In another in vitro study, a laser treated smooth surface resulted in significantly less *P. gingivalis* adhesion than the more rough sandblasted and machined surfaces (Di Giulio et al., 2015). On the other hand, larger numbers of periodontopathogens have been observed on titanium than on zirconia abutments after three months, despite that there were no differences in surface roughness (van Brakel et al., 2011). Further, it has been previously shown, *in vitro*, that titanium is less antibacterial than gold, but has a higher antibacterial activity against selected oral species than, for example, vanadium and aluminium (Berry, Moore, Safar, Henry, & Wagner, 1992). Comparing adhesion to titanium and hydroxyapatite disks resulted in a species-specific effect with more *S. mutans* and less *A. naeslundii* on titanium than on hydroxyapatite irrespective if discs were coated with serum or not (Lima, Koo, Vacca Smith, Rosalen, & Del Bel Cury, 2008). Thus, the differences in bacterial adhesion observed herein may be also partly attributed to the material *per se.*

P. gingivalis gingipain mRNA expression was always higher on dentin comparing with titanium. Titanium apparently exerted an inhibitory effect on gingipain expression initially. However, expression of all three gingipains was high after 24h, and in particular at serum-coated titanium, and there were no significant differences between the two types of substrates.

Activity of Arg-gingipains correlated with adhered counts of *P. gingivalis* and was promoted by HSA, something that may be explained by the fact that Arg-gingipains degrade many serum proteins, including serum albumin, to provide a source of nutrients for the bacterium (Grenier et al., 2001). In the gingival sulcus, protein levels are up to 1.5 µM for Arg-gingipains (Guentsch et al., 2011) and 10 nM for Kgp (Guentsch et al., 2013). Gingipains have been shown to cleave cell adhesion molecules, e.g. Kgp cleaves integrin β1 essential for endothelial cell adhesion (Sheets, Potempa, Travis, Fletcher, & Casiano, 2006), both Rgp and Kgp are involved in the cleavage of intracellular adhesion molecule-1 (ICAM-1) leading to a disruption in the interaction between PMN cells and the oral epithelium (Tada et al., 2003). Gingipains interfere with innate and acquired immunity. All three gingipains can activate in low concentration complement system but when present in higher concentration they cleave factors of complement system (Popadiak, Potempa, Riesbeck, & Blom, 2007). In particular Kgp reduces CD14 expression leading to a hyporesponsiveness of macrophages (Wilensky, Tzach-Nahman, Potempa, Shapira, & Nussbaum, 2015). Moreover, Kgp cleaves heavy chains of immunoglobulin G1 and G3 (Vincents et al., 2011). Gingipains promote destruction of the surrounding tissues by activating matrix-metalloproteases (MMPs), *e.g.* Arg-gingipain activates MMP-2 (Grayson, Douglas, Heath, Rawlinson, & Evans, 2003). Kgp cleaves osteoprotegerin thus inducing osteoclastogenesis (Akiyama et al., 2014). Further, Arg-gingipains induce receptor activator of NF-κB ligand (RANKL) stimulating bone resorption (Belibasakis et al., 2007). Recently it was shown that RgpA and Kgp DNA vaccines retard bone loss in experimental peri-implantitis animal models (M. Guo et al., 2014). In this context, it needs to be pointed out that research on gingipains related to dental implants is in general very scarce although their virulence properties might be of relevance.

In contrast to gingipains, knowledge about *T. forsythia* proteases is more limited. In this study, mRNA expression of three recently identified proteases, i.e., miropsin-1, miropsin-2, and mirolase (Ksiazek, Mizgalska, Eick, et al., 2015) was assessed. It has been demonstrated before, that miropsins exhibit a low proteolytic activity against several protein substrates, while mirolase can degrade fibrinogen, hemoglobin, and the antimicrobial peptide LL-37 (Ksiazek, Karim, et al., 2015). Expression of all evaluated *T. forsythia* proteases was initially low irrespective the substrate; after 24 h, however, expression increased and was highest in the presence of serum.

Moreover, expression of miropin, a protease inhibitor was analyzed. Miropin was shown to block neutrophil serine proteases (elastase, cathepsin G), trypsin, the bacterial protease subtilisin, and may also play a role in regulation of *T. forsythia* endogenous proteases (Ksiazek, Mizgalska, Enghild, et al., 2015). In our study, expression of miropin was significantly

upregulated in dentin (25 times higher) compared with titanium, irrespective the presence of serum or not. This finding suggests that there may be differences in the virulence of *T. forsythia* and the host response around titanium surfaces in comparison with teeth.

In the present study, no significant differences in bacterial counts were found irrespective substrate or coating when a bacterial mixture consisting of six strains was used. This set-up may mimic slightly closer the in vivo situation, since the biofilms on teeth or on dental implants are not single-species biofilms, but consist of more than hundred genera (Schaumann et al., 2014). Indeed, bacterial counts after 24 h in 6-species biofilms exceeded those of each single strain underlining the importance of the other included species in biofilm formation. On teeth oral streptococci are well known as primarily colonizers, thereafter other species e.g. *Actinomyces* sp. are recruited (Jakubovics & Kolenbrander, 2010). *F. nucleatum* acts as a bridging species adhering to early colonizers and providing receptors for late colonizers, e.g. *P. gingivalis* (Kolenbrander & London, 1993). Metabolic interactions and signaling molecules contribute to maturation of biofilms (Jakubovics & Kolenbrander, 2010). It might thus be of interest to study further interactions between several species not only related to tooth surfaces but also to implants.

Conclusion

The present findings indicate that: a) single bacterial species (*T. forsythia*) can adhere more readily to titanium than to dentin and adhesion may be influenced by serum proteins, b) low expression of *T. forsythia* protease inhibitor may influence the virulence of the species on titanium surfaces in comparison with teeth, and c) surface properties (e.g. material and/or protein layers) do not appear to significantly influence multi-species adhesion.

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References

Akiyama, T., Miyamoto, Y., Yoshimura, K., Yamada, A., Takami, M., Suzawa, T., . . . Kamijo, R. (2014). Porphyromonas gingivalis-derived lysine gingipain enhances osteoclast differentiation induced by tumor necrosis factor-alpha and interleukin-1beta but suppresses that by interleukin-17A: importance of proteolytic degradation of osteoprotegerin by lysine gingipain. *J Biol Chem,* 289(22), 15621-15630.

Arikan, F., Buduneli, N., & Lappin, D. F. (2011). C-telopeptide pyridinoline crosslinks of type I collagen, soluble RANKL, and osteoprotegerin levels in crevicular fluid of dental implants with peri-implantitis: a case-control study. *The International Journal of Oral & Maxillofacial Implants,* 26(2), 282-289.

Badihi Hauslich, L., Sela, M. N., Steinberg, D., Rosen, G., & Kohavi, D. (2013). The adhesion of oral bacteria to modified titanium surfaces: role of plasma proteins and electrostatic forces. *Clinical Oral Implants Research,* 24 Suppl A100, 49-56.

Belibasakis, G. N., Bostanci, N., Hashim, A., Johansson, A., Aduse-Opoku, J., Curtis, M. A., & Hughes, F. J. (2007). Regulation of RANKL and OPG gene expression in human gingival fibroblasts and periodontal ligament cells by Porphyromonas gingivalis: a putative role of the Arg-gingipains. *Microbial Pathogenesis,* 43(1), 46-53.

Berry, C. W., Moore, T. J., Safar, J. A., Henry, C. A., & Wagner, M. J. (1992). Antibacterial activity of dental implant metals. *Implant Dentistry,* 1(1), 59-65.

Buser, D., Janner, S. F., Wittneben, J. G., Bragger, U., Ramseier, C. A., & Salvi, G. E. (2012). 10-year survival and success rates of 511 titanium implants with a sandblasted and acid-etched surface: a retrospective study in 303 partially edentulous patients. *Clin Implant Dent Relat Res,* 14(6), 839-851.

Canullo, L., Penarrocha-Oltra, D., Covani, U., Botticelli, D., Serino, G., & Penarrocha, M. (2016). Clinical and microbiological findings in patients with peri-implantitis: a cross-sectional study. *Clin Oral Implants Res,* 27(3), 376-382.

Carlen, A., Rudiger, S. G., Loggner, I., & Olsson, J. (2003). Bacteria-binding plasma proteins in pellicles formed on hydroxyapatite in vitro and on teeth in vivo. *Oral Microbiology and Immunology,* 18(4), 203-207.

Choi, S., Baik, J. E., Jeon, J. H., Cho, K., Seo, D. G., Kum, K. Y., . . . Han, S. H. (2011). Identification of Porphyromonas gingivalis lipopolysaccharide-binding proteins in human saliva. *Molecular Immunology,* 48(15-16), 2207-2213.

Di Giulio, M., Traini, T., Sinjari, B., Nostro, A., Caputi, S., & Cellini, L. (2015). Porphyromonas gingivalis biofilm formation in different titanium surfaces, an in vitro study. *Clinical Oral Implants Research*.

Eick, S., Straube, A., Guentsch, A., Pfister, W., & Jentsch, H. (2011). Comparison of real-time polymerase chain reaction and DNA-strip technology in microbiological evaluation of periodontitis treatment. *Diagnostic Microbiology and Infectious Disease,* 69(1), 12-20.

Frohlich, E., Kantyka, T., Plaza, K., Schmidt, K. H., Pfister, W., Potempa, J., & Eick, S. (2013). Benzamidine derivatives inhibit the virulence of Porphyromonas gingivalis. *Molecular Oral Microbiology,* 28(3), 192-203.

Furst, M. M., Salvi, G. E., Lang, N. P., & Persson, G. R. (2007). Bacterial colonization immediately after installation on oral titanium implants. *Clinical Oral Implants Research,* 18(4), 501-508.

Goodson, J. M. (2003). Gingival crevice fluid flow. *Periodontology 2000,* 31, 43-54.

Grayson, R., Douglas, C. W., Heath, J., Rawlinson, A., & Evans, G. S. (2003). Activation of human matrix metalloproteinase 2 by gingival crevicular fluid and Porphyromonas gingivalis. *Journal of Clinical Periodontology,* 30(6), 542-550.

Grenier, D., Imbeault, S., Plamondon, P., Grenier, G., Nakayama, K., & Mayrand, D. (2001). Role of gingipains in growth of Porphyromonas gingivalis in the presence of human serum albumin. *Infection and Immunity,* 69(8), 5166-5172.

Griffiths, G. S. (2003). Formation, collection and significance of gingival crevice fluid. *Periodontology 2000,* 31, 32-42.

Guentsch, A., Hirsch, C., Pfister, W., Vincents, B., Abrahamson, M., Sroka, A., . . . Eick, S. (2013). Cleavage of IgG1 in gingival crevicular fluid is associated with the presence of Porphyromonas gingivalis. *Journal of Periodontal Research,* 48(4), 458-465.

Guentsch, A., Kramesberger, M., Sroka, A., Pfister, W., Potempa, J., & Eick, S. (2011). Comparison of gingival crevicular fluid sampling methods in patients with severe chronic periodontitis. *Journal of Periodontology,* 82(7), 1051-1060.

Guo, M., Wang, Z., Fan, X., Bian, Y., Wang, T., Zhu, L., & Lan, J. (2014). kgp, rgpA, and rgpB DNA vaccines induce antibody responses in experimental peri-implantitis. *Journal of Periodontology,* 85(11), 1575-1581.

Guo, Y., Nguyen, K. A., & Potempa, J. (2010). Dichotomy of gingipains action as virulence factors: from cleaving substrates with the precision of a surgeon's knife to a meat chopper-like brutal degradation of proteins. *Periodontology 2000,* 54(1), 15-44.

Hagi, T. T., Klemensberger, S., Bereiter, R., Nietzsche, S., Cosgarea, R., Flury, S., . . . Eick, S. (2015). A Biofilm Pocket Model to Evaluate Different Non-Surgical Periodontal Treatment Modalities in Terms of Biofilm Removal and Reformation, Surface Alterations and Attachment of Periodontal Ligament Fibroblasts. *PLoS One,* 10(6), e0131056.

Hajishengallis, G., Darveau, R. P., & Curtis, M. A. (2012). The keystone-pathogen hypothesis. *Nature Reviews. Microbiology,* 10(10), 717-725.

Jakubovics, N. S., & Kolenbrander, P. E. (2010). The road to ruin: the formation of disease-associated oral biofilms. *Oral Diseases,* 16(8), 729-739.

Kim, H., Choi, S. H., Ryu, J. J., Koh, S. Y., Park, J. H., & Lee, I. S. (2008). The biocompatibility of SLA-treated titanium implants. *Biomedical Materials,* 3(2), 025011.

Kindblom, C., Davies, J. R., Herzberg, M. C., Svensater, G., & Wickstrom, C. (2012). Salivary proteins promote proteolytic activity in Streptococcus mitis biovar 2 and Streptococcus mutans. *Molecular Oral Microbiology,* 27(5), 362-372.

Kolenbrander, P. E., & London, J. (1993). Adhere today, here tomorrow: oral bacterial adherence. *J Bacteriol,* 175(11), 3247-3252.

Ksiazek, M., Karim, A. Y., Bryzek, D., Enghild, J. J., Thogersen, I. B., Koziel, J., & Potempa, J. (2015). Mirolase, a novel subtilisin-like serine protease from the periodontopathogen Tannerella forsythia. *Biological Chemistry,* 396(3), 261-275.

Ksiazek, M., Mizgalska, D., Eick, S., Thogersen, I. B., Enghild, J. J., & Potempa, J. (2015). KLIKK proteases of Tannerella forsythia: putative virulence factors with a unique domain structure. *Front Microbiol,* 6, 312.

Ksiazek, M., Mizgalska, D., Enghild, J. J., Scavenius, C., Thogersen, I. B., & Potempa, J. (2015). Miropin, a novel bacterial serpin from the periodontopathogen Tannerella forsythia, inhibits a broad range of proteases by using different peptide bonds within the reactive center loop. *J Biol Chem,* 290(1), 658-670.

Kumar, P. S., Mason, M. R., Brooker, M. R., & O'Brien, K. (2012). Pyrosequencing reveals unique microbial signatures associated with healthy and failing dental implants. *Journal of Clinical Periodontology,* 39(5), 425-433.

Lee, K. H., Tanner, A. C., Maiden, M. F., & Weber, H. P. (1999). Pre- and post-implantation microbiota of the tongue, teeth, and newly placed implants. *Journal of Clinical Periodontology,* 26(12), 822-832.

Lee, S. Y., Kim, K. K., & Choe, S. J. (2001). Binding of oral streptococci to human fibrinogen. *Oral Microbiol Immunol,* 16(2), 88-93.

Lima, E. M., Koo, H., Vacca Smith, A. M., Rosalen, P. L., & Del Bel Cury, A. A. (2008). Adsorption of salivary and serum proteins, and bacterial adherence on titanium and zirconia ceramic surfaces. *Clinical Oral Implants Research,* 19(8), 780-785.

Ozcakir-Tomruk, C., Chiquet, M., & Mericske-Stern, R. (2012). Tenascin-C and matrix metalloproteinase-9 levels in crevicular fluid of teeth and implants. *Clinical Implant Dentistry and Related Research,* 14(5), 672-681.

Park, W., Park, Y., Park, H., Yoo, S., Chung, S., Han, J., . . . Kim, D. M. (2015). A 10-year retrospective radiographic study of implantium dental implants. *Int J Periodontics Restorative Dent,* 35(1), 49-54.

Popadiak, K., Potempa, J., Riesbeck, K., & Blom, A. M. (2007). Biphasic effect of gingipains from Porphyromonas gingivalis on the human complement system. *Journal of Immunology,* 178(11), 7242-7250.

Quirynen, M., Bollen, C. M., Papaioannou, W., Van Eldere, J., & van Steenberghe, D. (1996). The influence of titanium abutment surface roughness on plaque accumulation and gingivitis: short-term observations. *The International Journal of Oral & Maxillofacial Implants,* 11(2), 169-178.

Quirynen, M., Vogels, R., Peeters, W., van Steenberghe, D., Naert, I., & Haffajee, A. (2006). Dynamics of initial subgingival colonization of 'pristine' peri-implant pockets. *Clinical Oral Implants Research,* 17(1), 25-37.

Sanchez-Siles, M., Lucas-Azorin, J., Salazar-Sanchez, N., Carbonell-Meseguer, L., & Camacho-Alonso, F. (2016). Salivary Concentration of Oxidative Stress Biomarkers in a Group of Patients with Peri-Implantitis: A Transversal Study. *Clin Implant Dent Relat Res,* 18(5), 1015-1022.

Schaumann, S., Staufenbiel, I., Scherer, R., Schilhabel, M., Winkel, A., Stumpp, S. N., . . . Stiesch, M. (2014). Pyrosequencing of supra- and subgingival biofilms from inflamed peri-implant and periodontal sites. *BMC Oral Health,* 14, 157.

Sheets, S. M., Potempa, J., Travis, J., Fletcher, H. M., & Casiano, C. A. (2006). Gingipains from Porphyromonas gingivalis W83 synergistically disrupt endothelial cell adhesion and can induce caspase-independent apoptosis. *Infection and Immunity,* 74(10), 5667-5678.

Siegrist, B. E., Brecx, M. C., Gusberti, F. A., Joss, A., & Lang, N. P. (1991). In vivo early human dental plaque formation on different supporting substances. A scanning electron microscopic and bacteriological study. *Clinical Oral Implants Research,* 2(1), 38-46.

Socransky, S. S., Haffajee, A. D., Cugini, M. A., Smith, C., & Kent, R. L., Jr. (1998). Microbial complexes in subgingival plaque. *Journal of Clinical Periodontology,* 25(2), 134-144.

Tada, H., Sugawara, S., Nemoto, E., Imamura, T., Potempa, J., Travis, J., . . . Takada, H. (2003). Proteolysis of ICAM-1 on human oral epithelial cells by gingipains. *Journal of Dental Research,* 82(10), 796-801.

Tew, J. G., Marshall, D. R., Burmeister, J. A., & Ranney, R. R. (1985). Relationship between gingival crevicular fluid and serum antibody titers in young adults with generalized and localized periodontitis. *Infection and Immunity,* 49(3), 487-493.

Tomek, M. B., Neumann, L., Nimeth, I., Koerdt, A., Andesner, P., Messner, P., . . . Schaffer, C. (2014). The S-layer proteins of Tannerella forsythia are secreted via a type IX secretion system that is decoupled from protein O-glycosylation. *Molecular Oral Microbiology,* 29(6), 307-320.

van Brakel, R., Cune, M. S., van Winkelhoff, A. J., de Putter, C., Verhoeven, J. W., & van der Reijden, W. (2011). Early bacterial colonization and soft tissue health around zirconia and titanium abutments: an in vivo study in man. *Clinical Oral Implants Research,* 22(6), 571-577.

Vincents, B., Guentsch, A., Kostolowska, D., von Pawel-Rammingen, U., Eick, S., Potempa, J., & Abrahamson, M. (2011). Cleavage of IgG1 and IgG3 by gingipain K from Porphyromonas gingivalis may compromise host defense in progressive periodontitis. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 25(10), 3741-3750.

Wilensky, A., Tzach-Nahman, R., Potempa, J., Shapira, L., & Nussbaum, G. (2015). Porphyromonas gingivalis gingipains selectively reduce CD14 expression, leading to macrophage hyporesponsiveness to bacterial infection. *Journal of Innate Immunity,* 7(2), 127-135.

Figure legends

Fig. 1. Adhesion (colony forming units (CFU)) of *Porphyromonas gingivalis* ATCC 33277 (A) incl. its arginine-specific amidolytic acitivity (B), and *Tannerella forsythia* ATCC 43037 (C) to dentine and titanium surfaces coated with 0.9% w/v NaCl, human saliva, 25% human serum, 10 mg/ml human serum albumin (HSA), 5 mg/ml immunoglobulin G (IgG) after 4 h and 24 h of incubation

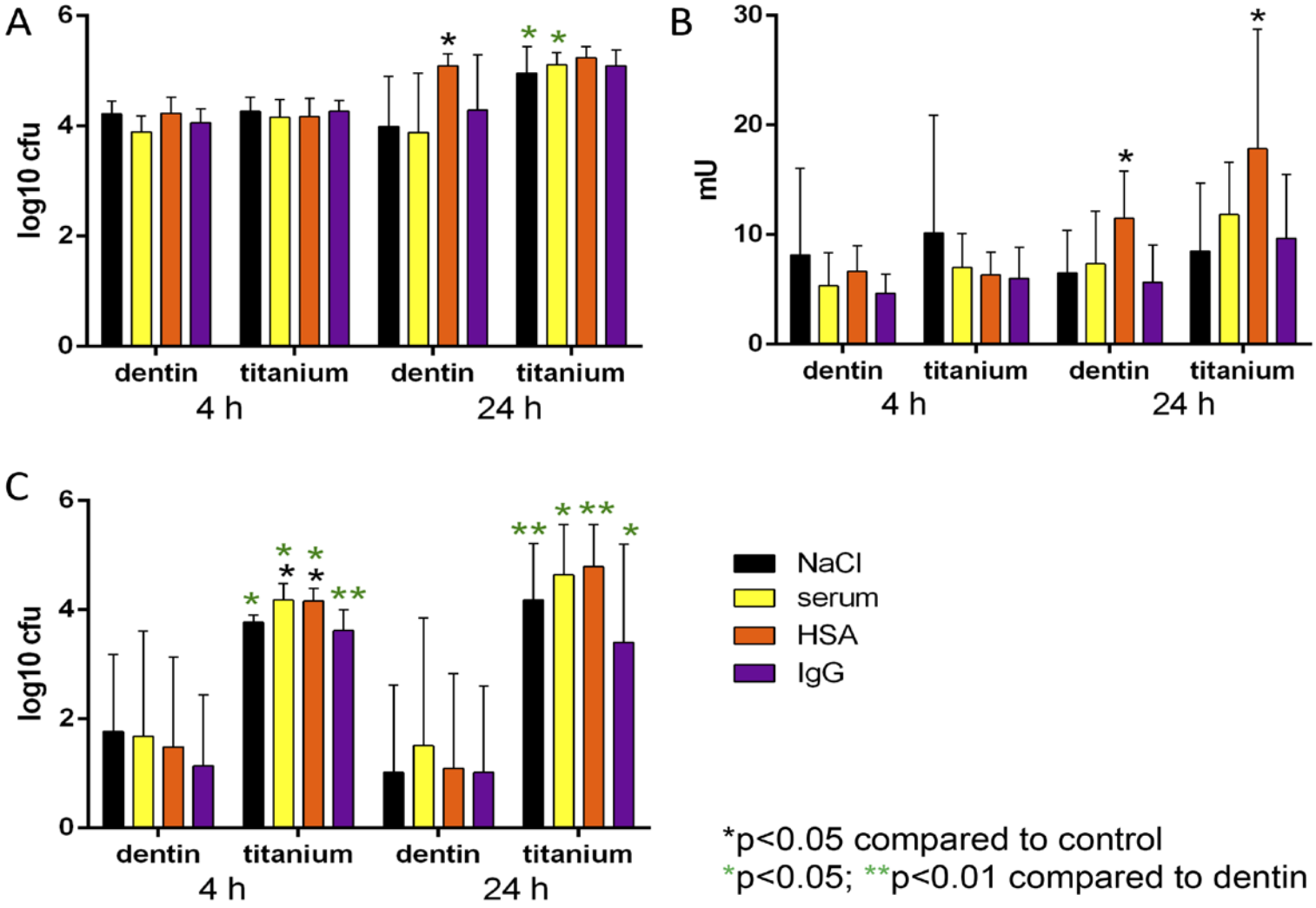
Fig. 2. Adhesion of six-species mixture to dentine and titanium surfaces coated with 0.9% w/v NaCl, 25% human serum, 10 mg/ml human serum albumin (HSA), 5 mg/ml immunoglobulin G (IgG) after 4 h and 24 h of incubation (A: total counts (CFU), B: counts of *Porphyromonas gingivalis* ATCC 33277 (real-time PCR), C: counts of *T. forsythia* ATCC 43037 (real-time PCR) D: arginine-specific amidolytic acitivity)

Fig. 3. Expression of *Porphyromonas gingivalis* proteases (mRNA expression related to *sod* and adjusted to planktonic bacteria before adhesion) after adhesion to dentine and titanium surfaces coated with 0.9% w/v NaCl and 25% human serum after 4 h and 24 h of incubation (A: *rgpA*, B: *rgpB*, C: *kgp*)

Fig. 4. Expression of *Tannerella forsythia* proteases and the protease inhibitor miropin (mRNA expression related to *sod* and adjusted to planktonic bacteria before adhesion) after adhesion to dentine and titanium surfaces coated with 0.9% w/v NaCl and 25% human serum after 4 h and 24 h of incubation (A: *miropsin-1*, B: *miropsin-2*, C: *mirolase*, D: *miropin*)

Table 1
Primer pairs used in real-time PCR for detection of bacterial proteases (*rgpA*, *rgpB*, *kgp*, *miropsin-1*, *miropsin-2*, *mirolase*) and of *T. forsythia* protease inhibitor (*miropin*) as well as the house keeping genes (*sod*)

Species	Gene	Primer	Reference
P. gingivalis	*rgpA*	fwd: 5′-TAT CCT TCG TGA TGT GCG TG-3′ rev: 5′-GCT GTA ACG GGA GAA GCA AT-3′	(Frohlich et al., 2013)
	rgpB	fwd: 5′-CAT TCT CCT CTC TGT TGG GA-3′ rev: 5′-CGT AGG GGA TTT GAT CAG GA-3′	(Frohlich et al., 2013)
	kgp	fwd: 5′-TCA AGC AGT TCG ATG CAA GC -3′ rev: 5′-ACT TGG GTC AGT TCT TGT CC-3′	(Frohlich et al., 2013)
	sod	fwd: 5′-AAT TCC ACC ACG GTA AGC AC-3′ 5′- TTC TCG ATG GAC AGT TTG CC-3′	(Frohlich et al., 2013)
T. forsythia	*miropsin-1*	fwd: 5’-CGT GCG TGA AGA AGC CAT TA-3′ rev: 5’-AAC CCG GAT GTT CAT ACC CC-3′	according to (Ksiazek, Mizgalska, Eick, et al., 2015)
	miropsin-2	fwd: 5’-TCC TGA CCG ACC TGA TCA AA rev: 5’-TCG GCA TTG GAA ATT TCG GA-3′	according to (Ksiazek, Mizgalska, Eick, et al., 2015)
	mirolase	fwd: 5’-TGC CGC AAA TCA TAA TGG TA rev: 5’-GTC CAT CCC TTC CTT GAG TG-3′	according to (Ksiazek, Mizgalska, Eick, et al., 2015)
	miropin	fwd: 5‘-ATG CCT TTG CCT TCG ATC TG-3′ rev: 5‘-CTT CCC GTA GTG AAT GGC TG-3′	(Ksiazek, Mizgalska, Enghild, et al., 2015)
	sod	fwd: 5’-GCA CGT CTG TTC TGG TAA TCC-3′ rev: 5’-CCT GCA ATT CAA GCC TCA GA-3′	accession: JUET01000058.1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log10 cfu
6
4
2
0
dentin
titanium
dentin
titanium
4 h
24 h
B
mU
30
20
10
0
dentin
titanium
dentin
titanium
4 h
24 h
C
log10 cfu
6
4
2
0
dentin
titanium
dentin
titanium
4 h
24 h
NaCl
serum
HSA
IgG
*p<0.05 compared to control
*p<0.05; **p<0.01 compared to dentin

Fig. 1

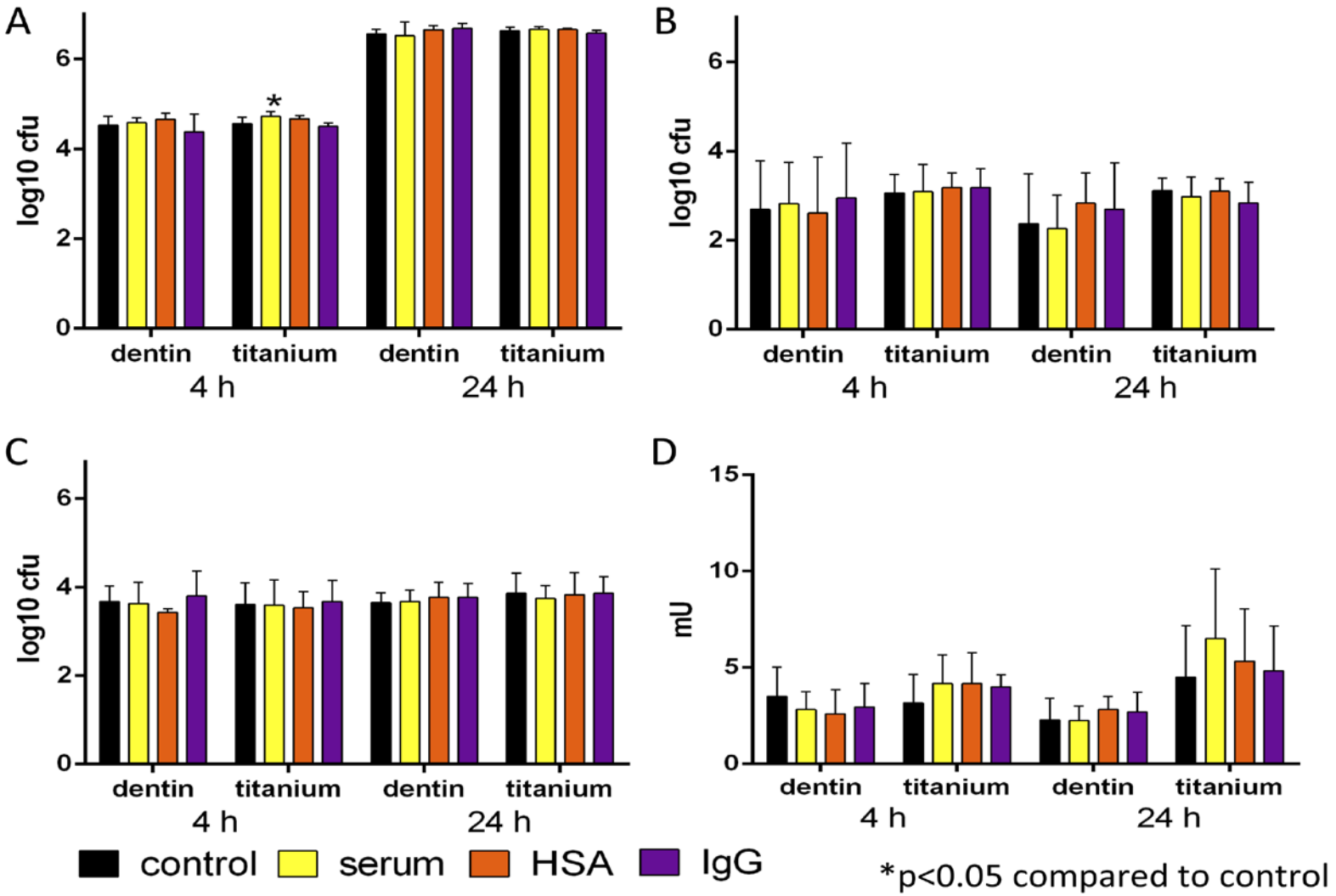
A
B
C
D
log10 cfu
mU
dentin
titanium
4 h
24 h
*
control
serum
HSA
IgG
*p<0.05 compared to control

Fig. 2

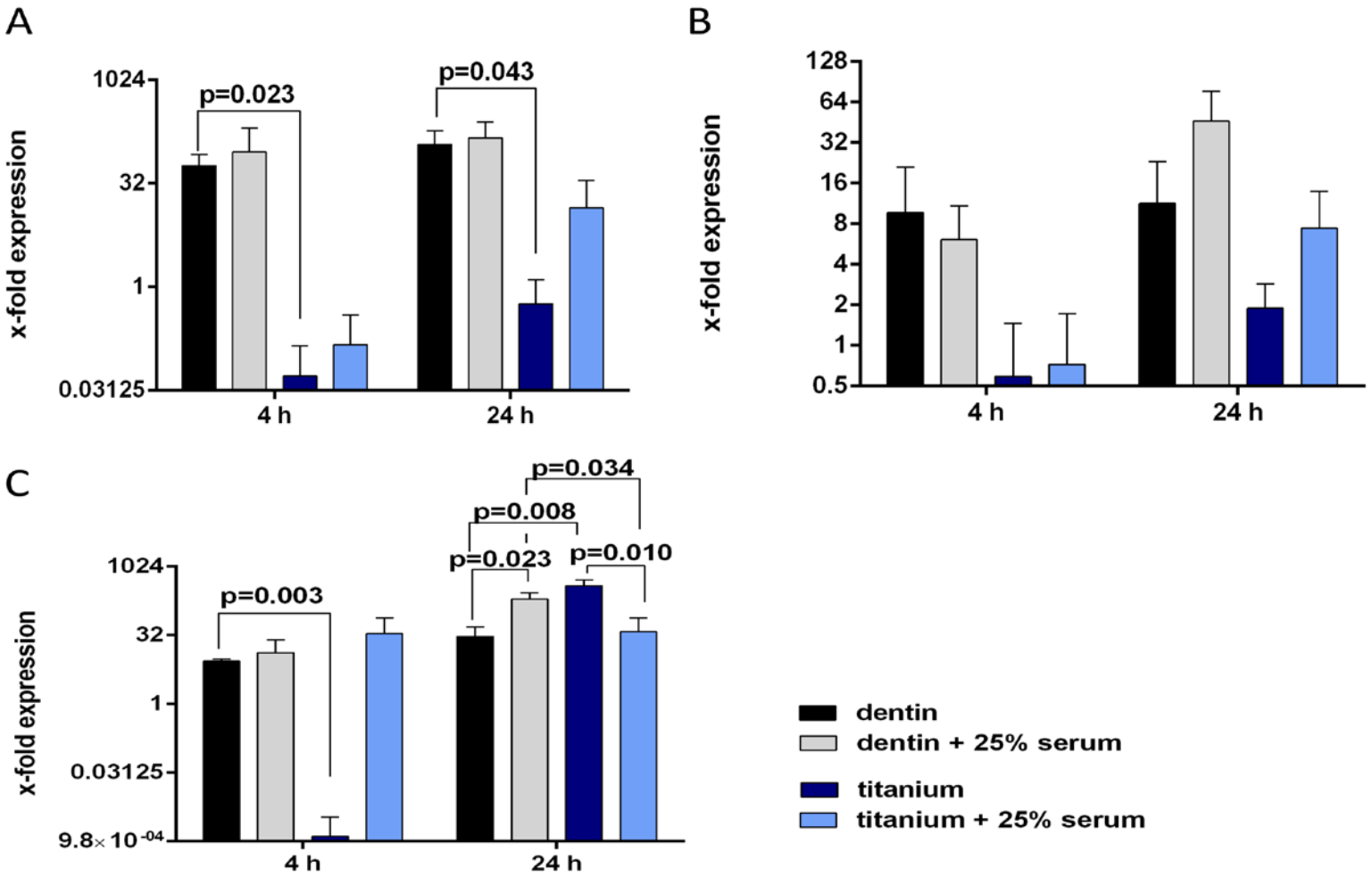
A
x-fold expression
1024
32
1
0.03125
p=0.023
p=0.043
4 h
24 h
B
x-fold expression
128
64
32
16
8
4
2
1
0.5
4 h
24 h
C
x-fold expression
1024
32
1
0.03125
9.8× 10 -04
p=0.003
p=0.034
p=0.008
p=0.023
p=0.010
4 h
24 h
dentin
dentin + 25% serum
titanium
titanium + 25% serum

Fig. 3

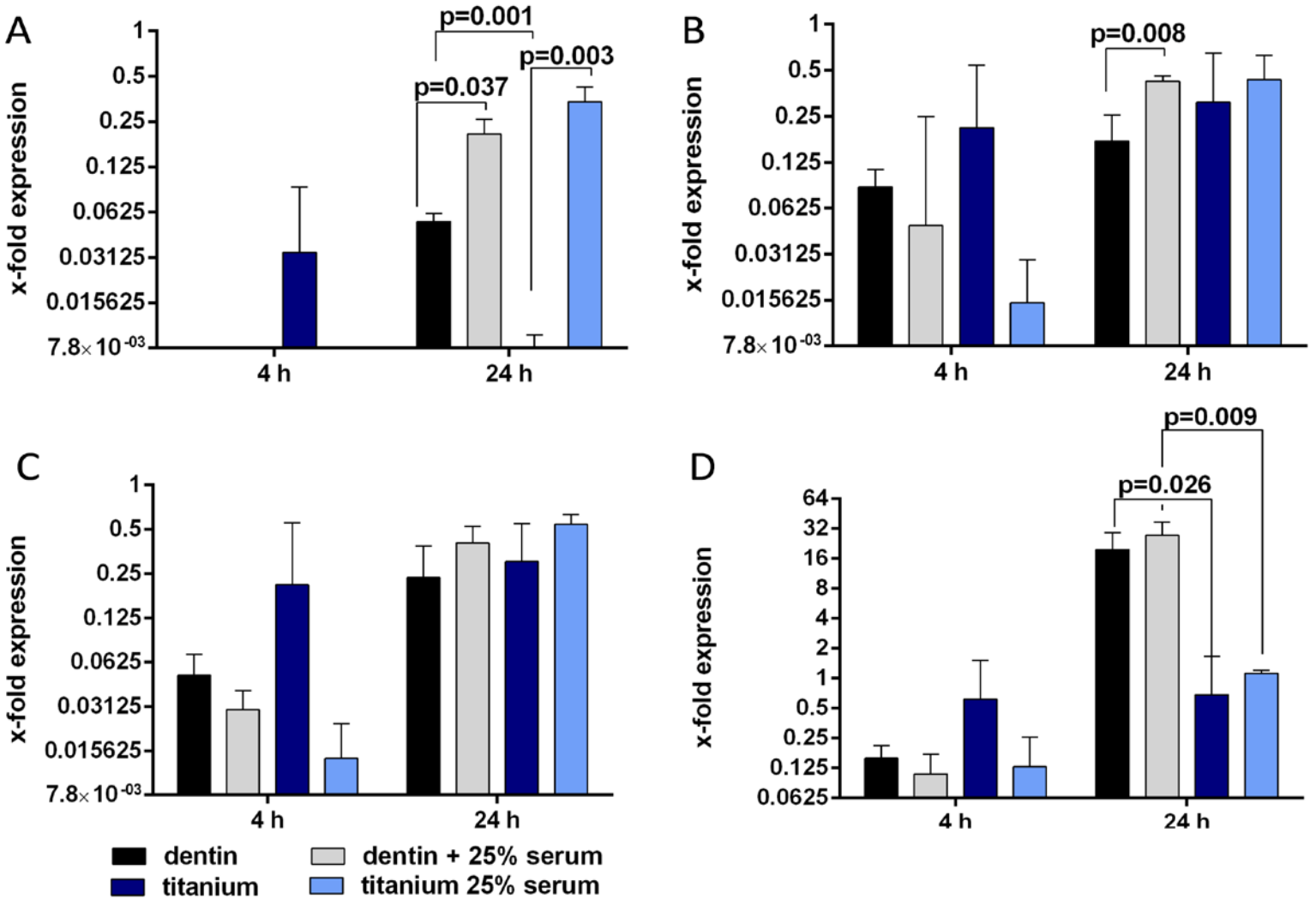
A
B
C
D
x-fold expression
1
0.5
0.25
0.125
0.0625
0.03125
0.015625
7.8× 10-03
4 h
24 h
p=0.001
p=0.003
p=0.037
p=0.008
p=0.009
p=0.026
64
32
16
8
4
2
1
dentin
titanium
dentin + 25% serum
titanium 25% serum

Fig. 4